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Involvement of Adhesion Molecules in Metastasis of SW1990, Human Pancreatic Cancer Cells

JIRO HOSONO, MD,[1] TATSUHIKO NARITA, MD,[2]* NAOKO KIMURA, PhD,[2] MIKINORI SATO, MD,[3] TATSUAKI NAKASHIO, MD,[1] YASUSHI KASAI, MD,[1] TOSHIAKI NONAMI, MD,[1] AKIMASA NAKAO, MD,[1] HIROSHI TAKAGI, MD,[1] AND REIJI KANNAGI, MD, PhD[2]

[1]*Department of Surgery II, Nagoya University School of Medicine, Nagoya, Japan*
[2]*Laboratory of Experimental Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan*
[3]*Department of Surgery I, Nagoya City University Medical School, Nagoya, Japan*

Background and Objective: Peritoneal dissemination and hepatic metastasis commonly occur after patients with pancreatic cancer have undergone surgery. It is thought that specific adhesion molecules play corresponding roles in cancer metastasis.

Study Design/Materials and Methods: We conducted in vitro and in vivo studies to assess the role of adhesion molecules in these processes, using SW1990 cells derived from human pancreatic cancer.

Results: SW1990 cells pronouncedly expressed sialyl Lewis^a (s-Le^a) and sialyl Lewis^x antigens (s-Le^x), CD44H, and β_1 integrin. Also, SW1990 cells showed a strong binding activity to IL-1β activated human umbilical vein endothelial cells, cultured murine endothelial cells (F-2 cells), and human peritoneal mesothelial cells. Invasive ability of SW1990 cells to F-2 cells was also observed. The adhesion leading to implantation of cancer cells to endothelial cells were inhibited by treatment with the antibodies against s-Le^a and against β1 integrin, respectively. Treatments with the antibodies against s-Le^a and β1 integrin each inhibited the development of liver metastasis in nude mice with SW1990 cells. The adhesion of SW1990 cells to peritoneal mesothelial cells was markedly inhibited by antibodies each against CD44 or β1 integrin, but was completely blocked by using a combination of these two antibodies. These antibodies inhibited the dissemination of SW1990 cells in the peritoneal cavity of nude mice and prolonged their survival.

Conclusion: These findings suggest that s-Le^a and integrin mediate the process from adhesion to implantation of SW1990 cells to endothelial cells, and CD44 and integrin play important roles in the initial attachment of SW1990 cells to mesothelial cells. It is thus speculated that compounds that interfere with the function of cell adhesion molecules may decrease the incidence of pancreatic cancer metastasis.

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KEY WORDS: **pancreatic cancer; metastasis; cell adhesion molecule; CD44; integrin; carbohydrate antigen**

INTRODUCTION

Peritoneal dissemination and hepatic metastasis commonly occur after patients with pancreatic cancer have undergone surgery. Therefore, the presence or absence of metastasis after surgery is one of the most critical factors

*Correspondence to: Tatsuhiko Narita, MD, Laboratory of Experimental Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464, Japan.

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in determining the prognosis of patients with pancreatic cancer. Recently, various cell adhesion molecules were found to be implicated in the metastasis of cancers [1]. We have already shown that the adhesion of cancer cells, including pancreatic cancer cells, to vascular endothelial cells is mediated by E-selectin and carbohydrate ligands, such as sialyl Lewis^a antigen (s-Le^a) and sialyl Le^x antigen (s-Le^x) [2,3]. However, the mechanisms by which pancreatic cancer cells bind to the peritoneal mesothelium have not yet been studied extensively. It is thought that specific adhesion molecules play corresponding roles in the adhesion of cancer cells to mesothelial cells. In the case of ovarian cancer, CD44 was shown partially to mediate the adhesion of tumor cells to peritoneal mesothelial cells [4–6]. Other reports have shown that this adhesion is inhibited by preincubating mesothelial cells with hyaluronic acid, which is a ligand for CD44 [7–9]. CD44 is an integral membrane glycoprotein that exists in a variety of forms with different molecular weights ranging from 85 Kd (CD44H) to 160 Kd (CD44E) [1, 10, 11]. Recent evidence suggests that CD44 participates in the invasion or metastatic process of cancer cells [1,11–14]. Thus, CD44 is thought to play certain roles in the peritoneal dissemination of pancreatic cancer cells. However, integrins are also involved in peritoneal dissemination, as they mediate the adhesion of cancer cells to extracellular matrix proteins.

We conducted both in vitro and in vivo investigations to assess the role of adhesion molecules in the attachment of pancreatic cancer cells to human vascular endothelial cells or mesothelial cells, which is the first step in the induction of distant metastasis. We further attempted metastasis inhibition by interfering with the functions of cell adhesion molecules that mediated the adhesion of pancreatic cancer cells to vascular endothelial cells or mesothelial cells in vivo.

MATERIALS AND METHODS

Cell Culture

SW1990 cells derived from human pancreatic cancer were kindly provided by Dr. T. Sawada (First Department of Surgery, Osaka City University, Osaka, Japan) [15] and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS) (Biowhittaker, MD). Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabou Co. (Osaka, Japan) and maintained in Daigo's T medium (Nissui Seiyaku, Tokyo), supplemented with 2 ng/ml of recombinant basic fibroblast growth factor (Takeda Pharmaceutical Co., Juso, Japan) and 10% FCS. The F-2 cells, which is a murine transformed cell line with tumorigenicity and vascular endothelial properties [16], were kindly provided by Dr. K.Toda (Department of Dermatology, Kyoto University, Kyoto, Japan) and were maintained in DMEM supplemented with 10% FCS. Human peritoneal mesothelial cells were taken from a patient with gastric cancer localized in the mucosa of the stomach. The separation of the mesothelial cells was done according to the method described previously [17,18]. Briefly, the resected omentum was incubated in a 0.25% trypsin solution for 20 min. After filtrating through an iron mesh, RPMI 1640 medium containing 10% FCS was added to the supernatant and then centrifuged. The pelletized material incorporating mesothelial cells was resuspended in medium and cultured until growth in the monolayers of mesothelial cells was seen. All the cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Reagents and Antibodies

Recombinant human interleukin-1β(rIL-1β) was kindly provided by Otsuka Pharmaceutical (Tokushima, Japan). The monoclonal antibodies (MAbs) SNH3 (specific to s-Le^x, supplied by Dr. Sen-itiroh Hakomori, Biomembrane Institute, Seattle, WA) and the 2D3 (specific to s-Le^a, established in our laboratory) are both murine IgM and were purified from ascitic fluids, as described previously [2,19]. The MAbs against CD44H and β1 integrin were obtained from Immunotech (Marseille, France) and Coulter Immunology (Hialeah, FL), respectively. The antimurine E-selectin MAb was kindly provided by Dr. D. Vestweber (Hans Spemann Laboratory, Max-Planck-Institute of Immunology, Freiburg, Germany) [20]. The antihuman E-selectin MAb was purchased from R&D Systems (Abington, Oxon, UK).

Flow Cytometric Analysis for Determination of Expression of Cell Surface Adhesion Molecules Expression

Flow cytometric analysis was performed using FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The indirect immunofluorescence method was applied to stain the cancer cells with various MAbs as the primary antibody (stained for 30 min at room temperature), followed by the addition of fluorescein isothiocyanate-labeled rabbit antimouse immunoglobulin (Silenus Lab, Hawthorn, Australia) as the secondary antibody.

Monolayer Cell Adhesion Assay Using Peritoneal Mesothelial Cells, HUVECs, and F-2 Cells

Monolayers of peritoneal mesothelial cells were grown in 24-well plates. To this, cancer cells (5×10^5 cells/well) were added and incubated for 60 min at 37°C. Monolayers of HUVEC and F-2 cell were read grown in 24-well plates. HUVECs were stimulated with 2 ng /ml of rIL-1β for 4 h in 24-well plates. To these, cancer cells (5×10^5 cells/well) were added and incubated under rotation (90 rpm) for 20 min at room temperature. After the nonadhering cells were washed out three times with PBS,

the number of attached cells were counted directly under a microscope. For the inhibition of cell adhesion, various MAbs (25 μm/well) were preincubated with cultured cancer cells for 30 min at room temperature prior to application on the monolayers of endothelial or mesothelial cells.

Evaluation of Implantation of Cancer Cells to Monolayers of F-2 Cell

Monolayers of F-2 cells were grown in 12-well plates. To these, cancer cells (1×10^5 cells/well) were added and co-cultured. After 24 h, they were placed under observation using a phase contrast microscope. For closer observation, cancer cells were cultured on F-2 cells monolayered on a slide glass, after which they were fixed with paraformaldehyde and stained with hematoxylin-eosin (H-E).

Raft culture was carried out according to previously described procedure [21] in order to obtain a vertical view. Briefly, eight volumes of type I collagen (Nitta Gelatin, Osaka, Japan), one volume of DMEM, and one volume of 0.05 N NaOH containing 200 mM Hepes and 260 mM NaHCO3 were mixed on ice and allowed to gel at 37°C in a 24-well plate. The F-2 cells were added onto the surface of the type I collagen gel and then cultured. When the F-2 cells reached confluence on the gel, the cancer cells were added. After co-cultivation for 24 h, they were fixed with a 10% formalin solution and detached from the well. Next, they were paraffin-embedded, sectioned, and stained with H-E.

Evaluations of Peritoneal Dissemination and Hepatic Metastasis of Cancer Cells in Nude Mice

Cancer cells (1×10^6 cells/100 μl RPMI 1640 medium, unless otherwise stated) were inoculated to the spleen or the intra-abdominal cavity of 5-week-old male BALB/c nu/nu nude mice (Charles River Japan Co., Yokohama). Cancer cells were preincubated with MAbs against CD44H (20 μg/mouse), β1 integrin (50 μg/mouse), s-Le^a (50 μg/mouse), and s-Le^x (50 μg/mouse) for 60 min prior to inoculation. In the peritoneal dissemination model, the mice were monitored for carcinomatous peritonitis until death. In the liver metastasis model, the mice were sacrified and checked for the presence of hepatic metastasis at 6 weeks after inoculation of the cancer cells to the spleen.

RESULTS

Expression of Cell Adhesion Molecules on Cancer Cells and Endothelial Cells

Figure 1 shows the expression of E-selectin on HUVECs and F-2 cells. Unstimulated HUVECs failed to express human E-selectin. However, when they were stimulated by rIL-1β for 4 h, expression of human E-selectin did occur. Expression of murine E-selectin, but not human E-selectin, was observed on unstimulated F-2 cells. Figure 2 shows the expression of adhesion molecules on SW1990 cells, using flow cytometric analysis. Pronounced expression of s-Le^a, s-Le^x, CD44H, and β1 integrin were observed.

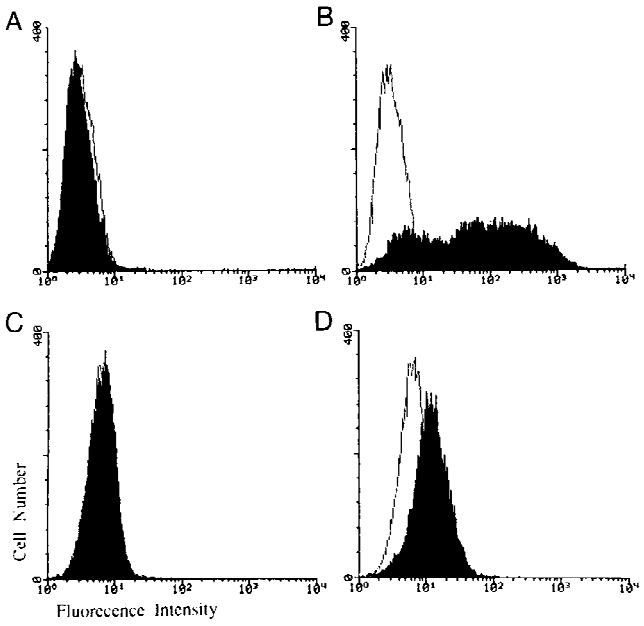

Fig. 1. Expression of E-selectin on HUVECs and F-2 cells. Unstimulated HUVECs failed to express human E-selectin **(A)**. When HUVECs were stimulated by rIL-1β, expression of human E-selectin was induced **(B)**. Unstimulated F-2 cells express murine E-selectin **(D)**, but not human E-selectin **(C)**.

Cell Adhesion Assay

The number of SW1990 cells adhering to unstimulated HUVECs was low. However, when subjected to monolayer cell-adhesion assay using rIL-1β-activated HUVECs, SW1990 cells exhibited a strong adhesion to HUVECs. SW1990 cells showed strong binding activity to F-2 cells, in the same way as activated HUVECs. The adhesion of SW1990 cells to activated HUVECs was inhibited significantly by the antibody against s-Le^a, but not by antibodies against s-Le^x, β1 integrin, or CD44H. Similarly, the adhesion of SW1990 cells to F-2 cells was markedly blocked by the addition of the antibody against s-Le^a (Figs. 3A–D,4).

SW1990 cells showed strong binding activity to human peritoneal mesothelial cells. This adhesion was partially inhibited by antibodies, respectively, against β1 integrin, or CD44H. However, almost complete inhibition was observed by the application of these two antibodies together. The antibodies against s-Le^x or s-Le^a were devoid of any detectable effect on the adhesion of SW1990 cells to mesothelial cells (Figs. 3E,F,5).

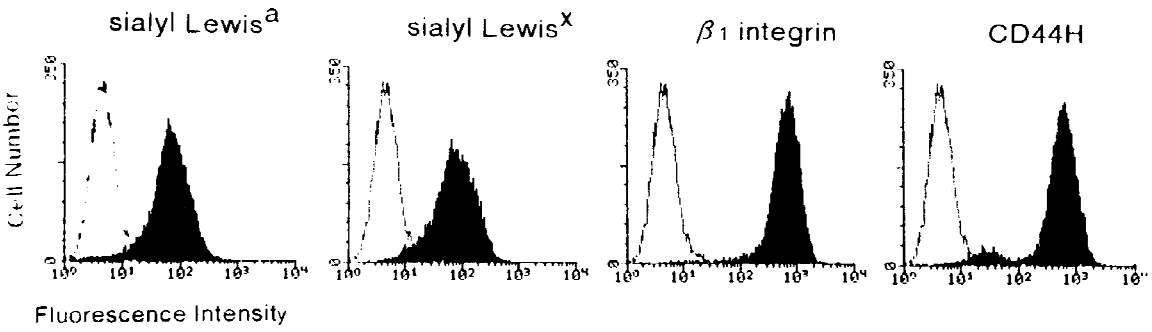

Fig. 2. Expression of adhesion molecules on SW1990 cells using flow cytometric analysis.

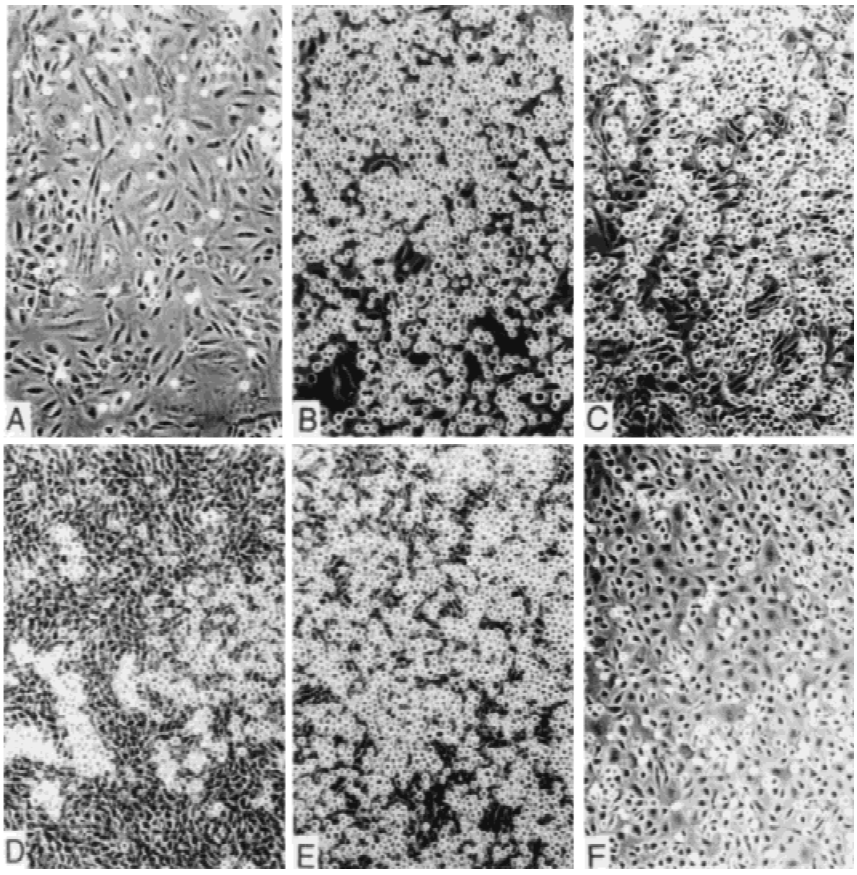

Fig. 3. Adhesion assay of SW1990 cells to monolayers of HUVECs, F-2 cells and peritoneal mesothelial cells. SW1990 cells adhere only weakly to nonactivated HUVECs **(A)**. When HUVECs are activated with IL-1β, adhesion of SW1990 cells to HUVECs is markedly accelerated **(B)**. SW1990 cells adhere to F-2 cells, the same as to activated HUVECs **(C)**. This adhesion is inhibited by treatment with anti-s-Le^a antibody **(D)**. SW1990 cells also show strong binding activity to peritoneal mesothelial cells **(E)**. This adhesion is inhibited by the addition of antibodies against CD44 and β1 integrin **(F)**.

Implantation of SW1990 Cells to Monolayers of F-2 Cells

When SW1990 cells were cultured on monolayers of F-2 cells, after 24 h it was found implanted below the monolayers of the F-2 cells. This implantation was inhibited by the addition of the antibody against β1 integrin, but not that against s-Le^a (Fig. 6).

Evaluation of Hepatic Metastasis of SW1990 Cells in Nude Mice

SW1990 cells demonstrated metastasis to the liver after inoculation to the spleen. Treatment with antibodies against s-Le^a and β1 integrin inhibited the metastasis of SW1990 cells to the liver, but the antibodies against s-Le^x and CD44H did not show this activity (Table I).

Evaluation of Peritoneal Dissemination of SW1990 Cells in Nude Mice

In all the mice tested, SW1990 cells spread within the abdominal cavity with bloody ascitic fluid after intraperitoneal inoculation. All the mice died within 6 weeks (Fig. 7A). Reducing the number of cells inoculated improved the survival of the mice. Survival was further improved by treatment with antibodies against either β1 integrin, or CD44H. However, a more pronounced effect was observed with treatment by a combination of these

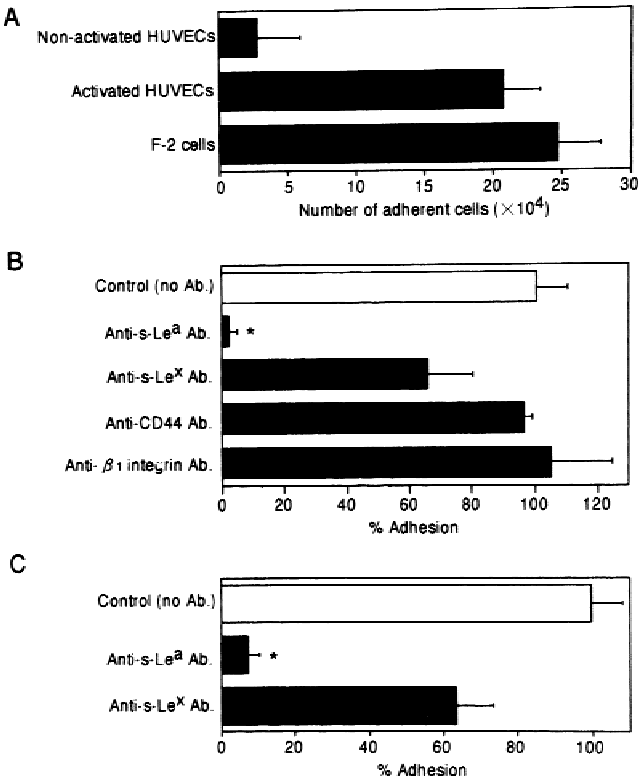

Fig. 4. Inhibitory effects of antibodies on the adhesion of SW1990 cells to HUVECs and to F-2 cells. SW1990 cells (5×10^5/well) were allowed to attach to nonactivated HUVECs, rIL-1β-activated HUVECs, and F2 cells for 20 min at room temperature with rotation (90 rpm) **(A)**. Inhibitory effects of various antibodies on the adhesion of SW1990 cells to activated HUVECs **(B)** and F-2 cells **(C)**. The number of adhering cells to rIL-1β-activated HUVECs or F-2 cells without antibody treatment is taken as control. The percentage of adhesion (abscissa) was calculated by comparing the number of adhering cells on each inhibitory experiment with those on the control. Bars indicate S.D. Asterisks represent differences from the control values that were shown to be statistically significant ($P<0.01$) by the Student's *t*-test.

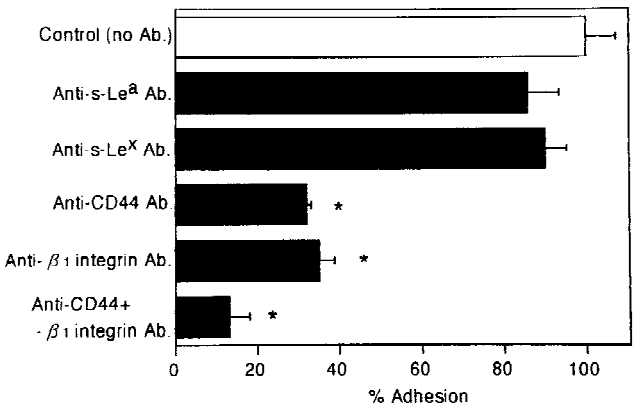

Fig. 5. Inhibitory effects of antibodies on the adhesion of SW1990 cells to peritoneal mesothelial cells. SW1990 cells (5×10^5/well) were allowed to attach to peritoneal mesothelial cells for 60 min at 37°C.

two antibodies, prolonging the mean survival time. Metastasis to the liver was also observed after intraperitoneal inoculation in all untreated mice. Thus treatment with antibodies against CD44H or β1 integrin inhibited not only peritoneal dissemination, but also hepatic metastasis. The antibody against s-Le^a inhibited the hepatic metastasis, but showed no detectable effect on peritoneal dissemination and survival of the mice (Fig. 7B and Table II).

DISCUSSION

Distant metastasis of pancreatic cancers take two major forms. One is hepatic metastasis and the other is peritoneal dissemination. It has been already reported that pancreatic cancer cells, including SW1990 cells, have the ability to effect hepatic metastasis in the splenic injection model [15]. However, only a few cell lines developed peritoneal metastasis in nude mice [22]. We investigated the ability of various cell lines derived from human gastointestinal cancers to effect hepatic and peritoneal metastasis. In the case of gastric cancer, we showed that NUGC-4 cells, which strongly express CD44, disseminated within the abdominal cavity of nude mice, and MKN74 cells, which strongly express s-Le^x, developed hepatic metastasis [23]. However, neither cell line had the ability to effect both metastases, as is the case with SW1990 cells.

It is widely accepted that gastric cancers of a poorly differentiated type (so-called scirrhous carcinoma) undergo peritoneal dissemination more frequently than the well-differentiated type. However, the well-differentiated type of gastric cancers often spread to the liver [22]. Thus the characteristics of individual tumors affects their metastatic form. In the case of pancreatic cancer, such difference has not yet been found. It is thought that SW1990 cells are suitable models reflecting the clinical features of pancreatic cancer.

The adhesion of SW1990 cells to human and cultured murine endothelial cell was inhibited by the antibody against s-Le^a. The adhesion of SW1990 cells to mesothelial cells was partly inhibited by antibodies against either CD44 or β1 integrin, but was completely blocked by a combination of these two antibodies. These findings suggest that s-Le^a mediates the adhesion of SW1990 cells to endothelial cells, and CD44 and β1 integrin mediate the adhesion of SW1990 cells to mesothelial cells. Thus it is thought that different adhesion molecules are separately involved in the adhesion of SW1990 cells to endothelial cells and to mesothelial cells. However, it is surmised that the extravasation of cancer cells, following initial adhesion of cancer cells to endothelial cells, is also an important step in the induction of hematogenous metastasis. SW1990 cells implanted to F-2 cells, but this was inhibited by the addition of anti-β1 integrin antibody. Yet, the mechanism of the invasion and implantation of cancer cells to endothelial cells remains unknown. In the case of the adhesion of leukocytes to endothelial

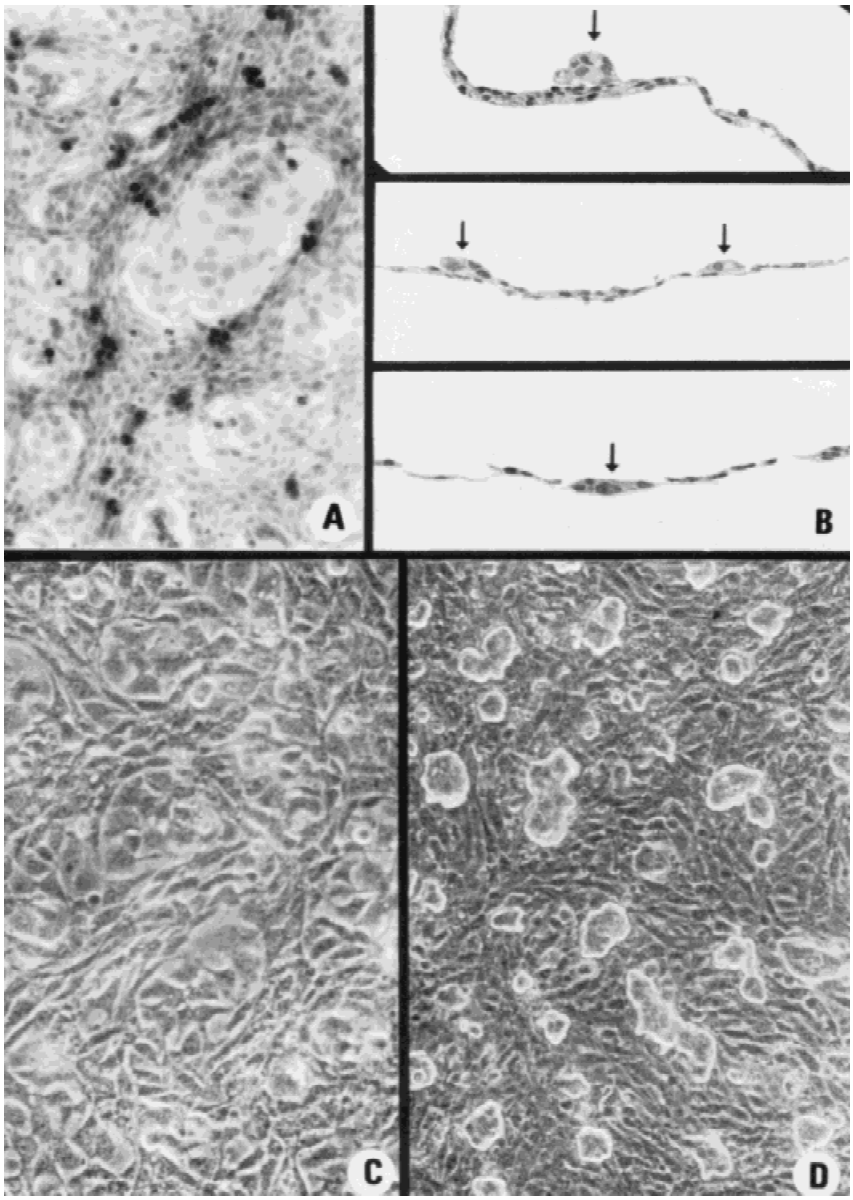

Fig. 6. Implantation of SW1990 cells to F-2 cells. SW1990 cells implant into the retractive space of F-2 cells (H-E staining, ×200) **(A)**. Findings by using raft culture is shown (×400) **(B)**. SW1990 cell (arrows) attach (upper panel), invade (median panel), and implant (lower panel) in F-2 cells. Implantation of SW1990 cells in monolayers of F-2 cells observed by using phase contrast microscopy (×200) **C**. This implantation is inhibited by addition of antibody against β1 integrin **(D)**.

cells, cell surface integrins are known to be activated following initial adhesion mediated by selectins and these activated integrins are implicated in the migration of leukocytes through vessel walls [24]. We reported previously that the activation of cell surface integrins induced by cytokines produced by endothelial cells, such as the heparin-binding EGF-like growth factor and hepatocyte growth factor, are involved in the transmigration of cancer cells to extravascular tisssues [25–27]. However, it is impossible to explain the entire process of implantation only by the function of adhesion molecules, such as integrin. In this process, the involvement of certain soluble factors, which are produced by cancer cells and which induce the retraction of endothelial cells, enhancing permeability, may be a possibility [28–30].

We also attempted metastasis inhibition by interfering

TABLE I. Inhibitory Effects of Antibodies on the Hepatic Metastasis of SW1990 Cells*

	Incidence[a]	No. of tumor nodules[b]
No Ab.	4/4	219.5 ± 22.5
anti-s-Le^X Ab.	4/4	212.0 ± 25.4
anti-s-Le^a Ab.	2/4	2.5 ± 3.8**
anti-CD44H Ab.	3/3	196.0 ± 16.9
anti-β_1 integrin Ab.	3/4	4.5 ± 4.2**

*Tumor take was assessed 6 weeks after intra-splenic inoculation. Ab.: antibody.
[a]Data are shown as the number of mice with hepatic metastasis/total number of mice.
[b]Mean ± S.D.
**Difference from control values is statistically significant at $P < 0.01$ by Student's *t*-test.

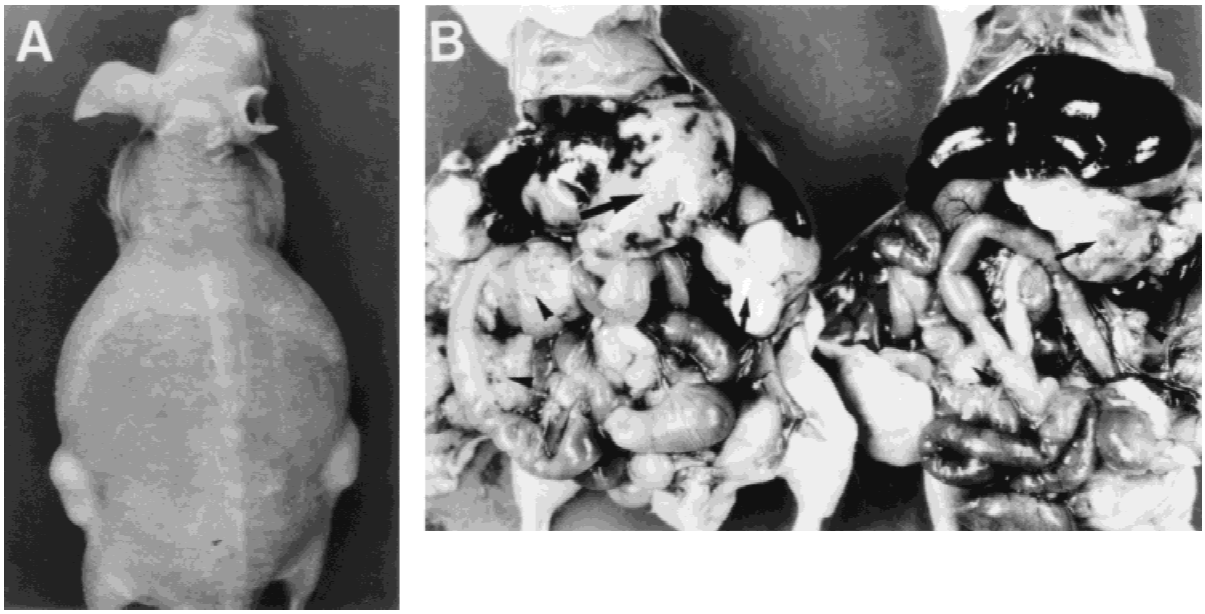

Fig. 7. Peritoneal dissemination of SW1990 cells in nude mice. Carcinomatous peritonitis was observed 4 weeks after the intraperitoneal inoculation of cancer cells. Abdominal distension revealed the presence of bloody ascites **(A)**. Peritoneal dissemination is seen as multiple white nodules on the peritoneum (arrowheads) and omentum (small arrows). Hepatic metastasis (large arrow) is also found in mice without treatment (left side). No hepatic metastasis is seen in mice treated with the antibody against s-Le[a] (right side) **(B)**.

TABLE II. Survival of Mice Inoculated With SW1990 Cells in the Abdominal Cavity and Improvement by Treatment With Various Antibodies

Inoculation	Number of inoculated cells	Number of mice	Mean ± S.D. of survival days after intraperitoneal
No Ab.[a] (Control)	1×10^6	5	30.2 ± 7.5
No Ab.	3×10^5	3	56.7 ± 22.3★
No. Ab.	1×10^5	3	89.3 ± 27.0★★
anti-s-Le[a] Ab.	1×10^6	3	40.7 ± 8.0
anti-CD44H Ab.	1×10^6	3	70.0 ± 17.4★
anti-β_1 integrin Ab.	1×10^6	3	89.0 ± 53.9★
anti-CD44H+ β_1 integrin Ab.	1×10^6	3	131.0 ± 71.9★★

[a]Ab.: antibody.
★★: difference is significant at $P < 0.01$ and ★: $P < 0.05$ by Student's *t*-test.

with the functions of cell adhesion molecules that mediated the adhesion of SW1990 cells to endothelial cells or mesothelial cells. SW1990 cells developed hepatic metastasis after intrasplenic inoculation, but this was inhibited by treatment with antibodies against s-Le[a] and β1 integrin. Dissemination of SW1990 cells in the abdominal cavity was observed after intraperitoneal inoculation, but this was inhibited by treatment with antibodies against CD44H and β1 integrin. We also investigated the cell toxicity of these antibodies and confirmed that these antibodies were not toxic to SW1990 cells by MTT assay (data not shown). Reducing the number of inoculated cells prolonged the mean survival term of nude mice in the peritoneal metastasis model. Therefore, we believe that the antiadhesive effects of antibodies on cancer cells and peritoneal mesothelial cells result in better prognoses. In the peritoneal metastasis model, hepatic metastasis was also observed, but this was inhibited by treatment not only with the antibodies against CD44H and β1 integrin, but also with the antibody against s-Le[a]. Consequently, it is thought that hepatic metastasis can occur through the peritoneum, but is inhibited by interfering with the functions of specific cell adhesion molecules. Similar results outlining the ability of antibodies against CD44 and β1 integrin to prevent tumor metastasis have been reported [31,32]. It is thus speculated that strategies to interfere with the function of cell adhesion molecules may decrease the incidence of the distant metastasis of pancreatic cancer and enhance the prognosis of patients with pancreatic cancer after surgery.

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